COMPOUNDS AND METHODS FOR IDENTIFYING COMPOUNDS WHICH INHIBIT A NEW CLASS OF ASPARTYL PROTEASES

Field of the Invention

A family of transmembrane protease enzymes has now been identified which comprise two critical aspartic acid residues
10 on the same side of the membrane at their active cleavage site. Members of this family of protease inhibitors can be identified by a consensus sequence at the C-terminal aspartic acid residue comprising G-X-G-D-(F or V or P or K or I or L or Y). The mechanism of action of these aspartyl proteases
15 is exemplified herein through the bacterial protease type 4 prepilin peptidase (TFPP). Accordingly, these proteases are referred to herein as TFPP-like aspartyl proteases. The present invention relates to compositions and methods for identifying and designing compositions which inhibit TFPP-like
20 aspartyl proteases by targeting the aspartic acid residues of the enzymatic active site or mimicking a region surrounding the cleavage site of a TFPP-like aspartyl protease substrate. These compositions are expected to be useful in inhibiting the activity of TFPP-like aspartyl proteases involved in diseases
25 and infections.

Background of the Invention

The development of the first anti-infective or anti-bacterial agents in the early 1900's and the success seen with their use led to the development of hundreds of new compounds

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to fight the wide variety of bacterial organisms. Most people
have had exposure to these drugs at some point in their life,
with the majority of situations resulting in a rapid cure of
the bacterial infection with relatively few side effects.
5 However, human morbidity and mortality due to bacterial
infections has become a major concern today due to the
dramatic increase in the frequency of infections caused by
bacteria that are resistant to most, if not all, of the
available anti-bacterial agents. This increase in drug-
10 resistant pathogens has led to an ever-increasing need for new
drugs with new mechanisms of action.

        Anti-bacterial agents are developed by identifying
unique targets not present in mammalian cells and then
designing a drug to exploit that difference such that the
15 bacterial cells are killed or neutralized while mammalian
cells are left intact and unaffected. The goal of successful
anti-bacterial drug therapy is to limit toxicity in the
patient while maximizing the ability of the drug to invade the
bacterial cells and neutralize those cells as selectively as
20 possible. The major classes of anti-bacterial drugs available
today target a variety of different cellular components and
functions of bacteria such as the cell wall, protein
synthesis, cell metabolism, DNA synthesis, and the bacterial
cell membrane. Each of these target cellular components or
25 functions is related in some way to the disease process of
bacterial infections that involves first colonization of the
bacteria, invasion of host cells, production of cellular
toxins or inflammatory agents, and a host response to those
toxins or agents.

30   A fundamental process of all living cells, including
bacteria, is the secretion of proteins across membranes. The
majority of proteins that are secreted are synthesized as a
precursor with an N-terminal signal sequence (or leader
peptide) of about 16-24 amino acids in length. This leader
35 sequence serves to promote recognition of the protein by the

secretory apparatus of the cell and facilitates movement across the membrane. The leader sequence is subsequently processed by a leader peptidase to remove the sequence and allow release of the mature or active protein. Recent

5 research has indicated that in the case of bacteria, there are several systems for secreting proteins and some of these systems have unique leader peptidases associated with their cognate secreted proteins. One of these systems is known as the type 2 secretion system which promotes extracellular

10 secretion of bacterial factors such as toxins and colonization pili that are the hallmarks of the mechanisms that promote virulence of pathogenic bacteria. Pili mediate the binding of bacteria to host tissues and most pili are composed of a major protein subunit that polymerizes to form a pilus.

15   The type 2 secretion systems of most bacteria involve a type 4 pilin for colonization pilus formation and type 4 pilin-like proteins for secretion of toxins and other factors associated with bacterial virulence and destruction of host tissue and enhancement of bacterial growth in the host.

20 Highly related type 4 pili serve as the major colonization factors for up to 50 different gram-negative bacterial species and type 4 pilin-like proteins have been found for a growing number of gram-positive bacteria as well. Type 4 pili are composed of a polymerized structure of type 4 pilin. The

25 pilin is synthesized as a prepilin with a leader peptide that is very different from those of typical secreted proteins. A type 4 specific leader peptidase is required to process a type 4 prepilin leader sequence to allow secretion of the mature protein. Importantly, this secretion system including

30 the type 4 leader peptidase itself is only found in bacteria and is not present in humans or other potential hosts of infection. Furthermore, mutating the type 4 prepilin peptidase (TFPP) renders the bacterium avirulent (March and Taylor 1998. *Mol. Microbiol.* 29:1481-1492).

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The type 4 signal peptide is highly conserved across all type 4 prepilin or prepilin-like proteins and is composed of 6 to 25 highly charged amino acids at the N-terminus followed by approximately 20 predominately hydrophobic amino acids.

5 Cleavage occurs between the two domains immediately C-terminal of an invariant glycine and before the new N-terminal amino acid that is usually a methionine or a phenylalanine. Unlike cleavage of standard signal peptide by signal peptidase I, wherein the cleavage occurs on the periplasmic side of the

10 inner membrane, processing by a type 4 peptidase occurs on the cytoplasmic side of the inner membrane (Strom and Lory, 1993. *Ann. Rev. Microbiol.* 47:565-596). Previous mutational analysis and protease inhibitor evidence from studies of *pilD* of *Pseudomonas aeruginosa* and protein alignment analysis of

15 the type 4 peptidase family suggested two pairs of cysteines in cytoplasmic domain 1, the largest cytoplasmic domain, to be involved in the protease active site of the enzyme (Strom et al. 1993. *Proc. Natl. Acad. Sci. USA* 90:2404-2408). These data resulted in the categorization of type 4 prepilin

20 peptidase (TFPP) family as a type of cysteine protease (Strom et al. 1994. *Meth. Enzymol.* 235:527-540).

However, subsequent studies have shown the two cysteine pairs to be lacking in XpsO, a type 4 prepilin peptidase of *X. campestris*. In fact, several type 4 prepilin peptidases,

25 among the approximately 27 that have been identified and cloned, do not have the conserved cysteines in their protein sequence but still function to cleave the type 4 signal peptide.

In the present invention a specific domain of type 4

30 prepilin peptidase that is essential for cleavage activity has now been identified to the resolution of two specific amino acids. Identification of this domain has facilitated the identification of specific inhibitors of the protease activity of type 4 prepilin peptidase as well as other TFPP-like

35 aspartyl proteases which utilize this same cleavage mechanism.

Summary of the Invention

Using mutant constructs of TcpJ, residues essential for
cleavage activity of the bacterial protease type 4 prepilin
peptidase (TFPP) have now been identified as two aspartic acid
5 residues. Thus, useful anti-bacterial agents targeted to
these residues can now be designed to inhibit the cleavage
activity of type 4 prepilin peptidase which is essential to
growth of bacteria in a host. Further, a consensus sequence
at the C-terminal aspartic acid residue comprising G-X-G-D-(F
10 or V or P or K or I or L or Y) has been identified so that
TFPP-like aspartyl proteases of organisms other than bacteria,
including humans, which comprise this novel active site and
utilize a cleavage mechanism similar to that exemplified
herein for type 4 prepilin peptidase can be identified. For
15 purposes of the present invention, proteases of this new
family which are transmembrane protease enzymes comprising two
critical aspartic acid residues on the same side of the
membrane at their active cleavage site, wherein a consensus
sequence at the C-terminal aspartic acid residue comprising
20 G-X-G-D-(F or V or P or K or I or L or Y) is present, are
referred to herein as "TFPP-like aspartyl proteases". This
term is meant to be inclusive of type 4 prepilin peptidases.

Accordingly, an object of the present invention is to
provide a method of designing inhibitors of TFPP-like aspartyl
25 proteases which comprises synthesizing compounds which target
the aspartic acid residues of the enzymatic active site of the
TFPP-like aspartyl protease or mimic a region surrounding the
cleavage site of the TFPP-like aspartyl protease substrate and
inhibit cleavage activity of the protease. Also provided in
30 the present invention are methods of selecting and screening
compounds for inhibitory activity of these TFPP-like aspartyl
protease enzymes. Compounds identified as inhibitors can be
used to modulate activity of TFPP-like aspartyl proteases
involved in disease and infection. In one embodiment,

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compounds identified as inhibitors are used as anti-bacterial agents in the inhibition of type 4 prepilin peptidases.

Thus, another object of the present invention is to provide new anti-bacterial agents which comprise compounds

5   which target the aspartic acid residues of type 4 prepilin peptidase and inhibit cleavage activity of this peptidase. As will be obvious to those of skill in the art upon this disclosure, such agents are also expected to be useful in inhibiting other TFPP-like aspartyl protease enzymes of this

10   family which utilize the same cleavage mechanism as described herein for type 4 prepilin peptidase and/or contain the same active site. Other TFPP-like aspartyl protease enzymes of this family can be identified routinely by those of skill in the art based upon the presence of the consensus sequence

15   comprising G-X-G-**D**-(F or V or P or K or I or L or Y) at the C-terminal aspartic acid residue of the enzymatic active site.

Another object of the present invention is to provide methods of inhibiting activity of TFPP-like aspartyl protease enzymes via administration of a compound which targets the

20   aspartic acid residues of the enzymatic active site of the TFPP-like aspartyl protease or mimics a region surrounding the cleavage site of the TFPP-like aspartyl protease substrate and inhibits cleavage activity of the protease. In one embodiment of the present invention, bacterial infection in a host is

25   inhibited by administering to a host infected with the bacteria a compound which targets the aspartic acid residues of type 4 prepilin peptidase and inhibits cleavage activity of this peptidase.

Also related to this embodiment of the present invention

30   are methods and compositions for decreasing development of drug resistant strains of bacteria through coadministration of a compound which targets the aspartic acid residues of type 4 prepilin peptidase and inhibits cleavage activity of this peptidase with a second known therapeutically effective anti-

35   bacterial agent.

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Another object of the present invention is to provide
TcpJ mutant constructs useful in defining the active cleavage
site of type 4 prepilin peptidase and in X-ray crystal
structure analysis of this enzyme.

5   Yet another object of the present invention is to
provide a homologous type 4 prepilin peptidase gene identified
in *Staphylococcus aureus*.

Brief Description of the Figures

Figure 1 is a bargraph showing the percent of conserved
10 homology of potential protease active site residues from 27
type 4 prepilin peptidase homologs. Conserved residues which
do not exist in TcpJ are in parenthesis and are designated
with the relative position in TcpJ. Predicted membrane
topology is indicated by C (cytoplasmic), P (periplasmic) or
15 M (transmembrane) below each residue. Shaded bars indicate
the conserved residues which were altered in experiments
described herein. Alignment was performed in DNASTAR MegAlign
using the Clustal method.

Figure 2 is a predicted TcpJ membrane topology model.
20 The predicted topology of TcpJ was deduced by comparison with
OutO of *Erwinia carotovora* for which the membrane topology has
been determined by the use of beta lactamase fusions in *outO*.
Cytoplasmic domains 1, 2 and 3 have been designated as well
as C (cytoplasmic), P (periplasmic) and M (transmembrane)
25 regions. All potential protease active sites (C, S, D, K, H,
E) have been designated. Amino acids altered in experiments
described herein are shaded and numbered by their position.
Each critical active site residue, D 125 and D189, is marked
with a "*".

30 **Detailed Description of the Invention**

The type 4 prepilin peptidase is found only in bacteria
and is responsible for the cleavage and N-methylation of a

large number of secreted proteins known as type 4 prepilins,
or prepilin-like proteins, that have a type 4 signal peptide
at their N-terminus. The cleavage of the type 4 signal
peptide is a necessary step to secretion of the type 4

5   prepilin. Prepilin-like proteins are involved in DNA
competence and/or uptake in *B. subtilis*, in phage
morphogenesis in *E. coli*, and partially comprise the type 2
secretion system which is the main terminal branch of the
general secretory pathway in gram negative bacteria (Pugsley,

10 1993. *Microbiology Reviews* 57(1):50-108). Type 4 pili serve
as the major colonization factors for up to 50 different gram-
negative bacterial species. Therefore, agents which interfere
with, or inhibit, activity of type 4 prepilin peptidase are
expected to be useful as anti-bacterial drugs and in the

15 identification of anti-bacterial drugs. Further, given the
role of prepilin-like proteins in DNA uptake, particularly in
gram-positive bacteria (Dubnau, D. 1997. *Gene* 192:191-198;
Wolfgang et al. 1999. *Molecular Microbiology* 31(5):1345-1357),
it is believed that coadministration of these agents with a

20 second anti-bacterial agent will inhibit uptake of
antimicrobial agent resistance gene thereby reducing
development of drug resistant strains of bacteria.

It has now been found that type 4 prepilin peptidase,
specifically TcpJ, a type 4 prepilin peptidase of *Vibrio*

25 *cholerae*, is a non-pepsin-like acid protease. A homologous
gene has also now been identified in *Staphylococcus aureus*,
a bacterium which is very difficult to treat due to its
ability to become resistant to drugs. The nucleic acid
sequence encoding this peptidase in *Staphylococcus aureus* is

30 depicted as SEQ ID NO:1. The amino acid sequence of this
Staphylococcus aureus peptidase is depicted as SEQ ID NO:2.
Contrary to prior art suggestions of cysteine residues being
involved in the protease active site of this enzyme ((Strom
et al. 1993. *Proc. Natl. Acad. Sci. USA* 90:2404-2408),

residues essential for cleavage activity of this peptidase
have now been identified as two aspartic acid residues.
Further, a consensus sequence at the C-terminal aspartic acid
residue comprising G-X-G-D-(F or V or P or K or I or L or Y)
5   has been identified so that TFPP-like aspartyl proteases of
organisms other than bacteria, including humans, which
comprise this novel active site and utilize a cleavage
mechanism similar to that exemplified herein for type 4
prepilin peptidase can be identified.   Accordingly, this
10   peptidase is part of a novel subclass or family of non-pepsin-
like acid proteases, referred to herein as TFPP-like aspartyl
proteases.   For purposes of the present invention, by "TFPP-
like aspartyl protease" it is meant a transmembrane protease
enzyme comprising two critical aspartic acid residues on the
15   same side of the membrane at its active cleavage site, wherein
a consensus sequence at the C-terminal aspartic acid residue
comprising G-X-G-D-(F or V or P or K or I or L or Y) is
present.   This term is meant to be inclusive of type 4
prepilin peptidases.

20   Further, it has now been demonstrated that agents which
target the aspartic acid residues of this active site inhibit
the cleavage activity of proteases in this new family.   For
example, inhibition of type 4 prepilin peptidase was
accomplished by contact of the peptidase with the combination
25   of   EDAC   (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
hydrochloride) and glycinamide, a combination of compounds
known to inhibit non-pepsin-like acid proteases.   Using the
knowledge of this target area within the peptidase sequence
essential for cleavage activity, additional inhibitors of this
30   class of protease enzymes can also be identified.   For
example, inhibitors designed to target the aspartic acid
residues, D125 and D189, which are essential to the cleavage
activity of type 4 prepilin peptidase, can be synthesized for
use as anti-bacterial agents.   Preferably, the inhibitors are
35   transition state analogues or other compounds that modify or

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competitively inhibit the aspartic acid residues thereby
preventing cleavage activity.

To determine the particular amino acids of aspartyl
proteases such as TcpJ and other type 4 prepilin peptidases
5 that act as the catalytic residues of the protease activity,
the protein sequence alignment of 27 type 4 prepilin
peptidases was examined for highly conserved amino acids which
have the potential to be protease active site residues. All
cysteines, serines, aspartic acids, histidines, lysines, and
10 glutamic acids that represent consensus residues in these type
4 prepilin peptidases are shown in Figure 1. Potential active
site residues of TcpJ, regardless of the level of
conservation, are also shown in Figure 1.

A mutational strategy was then used to determine the
15 active site residues of type 4 prepilin peptidase of *Vibrio
cholerae* (Genbank Accession No. M74708). The nucleic acid
sequence of this gene is depicted herein as SEQ ID NO:3; the
amino acid sequence of this peptidase is depicted as SEQ ID
NO:4. Fifteen of the most highly conserved active site
20 residues were mutated to either alanine or leucine in TcpJ,
the type 4 prepilin peptidase of *Vibrio cholerae.* The
residues selected included S18, S46, C48, C51, S65, C73, C76,
S81, E88, D125, S172, D183, D189, K191, S212, and S213 (see
Figure 2). D125 and D189 were also mutated to N and E. D183
25 was mutated to N. Mutations at the selected positions of *tcpJ*
were made in pCL10, a plasmid containing *tcpJ* and *tcpA*
expressed from a *lac* promoter. These mutant constructs of the
present invention were then introduced into *Vibrio cholerae*
CL381, a classical O395 with an insertional disruption in *tcpJ*
30 and an in-frame deletion of *vcpD* conferring a complete
inability to cleave TcpA. The resultant strains were grown
under TCP-inducing conditions (LB pH 6.5, 30°C). The
resulting whole cell extracts were loaded on a SDS-PAGE gel
and Western immunoblot was performed with antisera to TcpA.

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The results showed that D125A and D125N and D189A and D189N were the four mutants that displayed a complete loss of TcpJ activity. C48A and K191A showed some loss of activity while all other mutants did not show any loss of cleavage activity.

5 The chemical and structural requirements of the reactive sites were further defined by substituting E at positions D125 and D189. Partial activity was maintained. Since the chemical nature of the D and E side groups are similar, it is believed that the side group chemistry is responsible for the cleavage

10 activity of the peptidase. The chemical structure of D provides for the optimal reaction as compared with E.

E. coli amber suppressor strains were utilized to determine the activity of TcpJ peptidases with a number of amino acid substitutions at the 125 and 189 positions. An

15 amber (TAG) site was individually engineered into the 125 and 189 positions of the pCL10 plasmid and the resultant constructs were introduced into the Amber-Lys, Amber-Leu, Amber-Gln, Amber-Ser, Amber-Tyr amber suppressor strains. The strains were grown to mid-logarithmic phase and whole cell

20 extracts were examined for TcpA and TcpJ by Western immunoblot analysis. No amino acid substitution at either 125 or 189 exhibited any cleavage activity. The protein levels of TcpJ were all comparable to wild-type TcpJ.

TcpJ mutant activity was then assessed in an *in vitro*

25 assay modeled after the *in vitro* peptidase assay devised for PilD of *P. aeruginosa* (Nunn, D.N. and S. Lory. 1991. *Proc. Natl. Acad. Sci. USA* 88:3281-3285). Crude preparations of TcpA-containing membranes from JM109 p3Z-A and TcpJ-containing membranes from JM109 pCL9 were combined with cardiolipin and

30 5X assay buffer to commence cleavage of TcpA. After incubation of TcpA-containing membranes with approximately 0.5 μg of TcpA prepilin and increasing amounts of TcpJ-containing membranes, 1 unit of TcpJ activity was defined as the amount of TcpJ-containing membrane required to cleave 50% of 0.5 μg

35 of TcpA prepilin in 1 hour. To determine the *in vitro*

peptidase activity of 10 of the 16 TcpJ amino acid positions previously tested *in vivo*, single or multiple mutations representing each position were constructed in PCL9 and introduced into JM109. Membrane preparations of these *E. coli*

5 strains which lack TcpA prepilin were combined in the *in vitro* peptidase assay with 0.5 μg of TcpA prepilin from TcpA-containing membranes. Western blot analysis demonstrated that mutations at positions D125 and D189 were completely defective for processing whereas all other TcpJ mutants tested had some

10 peptidase activity except for C48A which showed no detectable cleavage activity. However, the C48A TcpJ protein degrades *in vitro*. The protein levels of all of the TcpJ mutants were comparable.

Since no residue was found in domain 1 that was an

15 absolute requirement for TcpJ cleavage activity and because several type 4 prepilin homologs do not have domain 1 in their protein sequence, the requirement for domain 1 of TcpJ for cleavage activity was examined. A deletion was made in domain 1 from position 35 to 81 of TcpJ using pCL11 producing

20 pCL11Δ35-81. The deletion of 35 through 81 removed the two cysteine pairs as well as the highly conserved serines at positions 46 and 65. This deletion therefore removes the residues that had been implicated in prepilin protease activity by PilD in *P. aeruginosa* (Strom and Lory, 1993.

25 *Ann. Rev. Microbiol.* 47:565-596). pCL11Δ35-81 was introduced into JM109 and a membrane preparation was made and tested in the *in vitro* cleavage assay. This assay demonstrated that the TcpJ derivative expressed by the deletion retained activity. Western analysis using an anti-6His antibody confirmed that

30 TcpJΔ35-81 was expressed and stable.

To determine the peptidase activity of TcpJΔ35-81 *in vivo*, pCL11Δ35-81 and the wild-type control pCL11 were introduced into J71K cells. The resultant strains were grown under TCP-inducing conditions. Whole cell extracts were

examined by SDS-PAGE and Western immunoblot analysis with anti-TcpA antisera. The results showed that pCL11Δ35-81 can complement J71K similar to pCL11. TcpJΔ35-81 was able to restore TCP biogenesis to J71K cells as seen by TEM with a

5   negative stain of the cultures. These data demonstrate that, contrary to prior art teachings (Strom, M.S. et al. 1993. *Proc. Natl. Acad. Sci. USA* 90:2404-2408), domain 1 is not required for type 4 prepilin cleavage or assembly of the resulting mature pilin into the pilus structure.

10   Thus, analysis of the protein sequence alignment of the type 4 prepilin peptidase family and the mutational strategy using the TcpJ mutant constructs coupled with the *in vivo* and *in vitro* assays confirmed that aspartic acid residues 125 and 189 make up the active site contrary to prior art teachings

15   relating to cysteine pairs.

            Based upon this identification, a standard TcpJ *in vitro* cleavage assay was performed with a number of chemical protease inhibitors to examine their ability to block TcpJ activity. Inhibitors were selected based upon this knowledge

20   of the active site that were expected to effect or not effect this peptidase activity. The effect of chemical protease inhibitors on the peptidase activity of TcpJ was determined by incubating the inhibitor with an amount of membrane preparation known to contain 1 unit of activity of TcpJ for

25   30 minutes at room temperature. The TcpJ/inhibitor mixture was then tested for peptidase activity in the in vitro processing assay described in Example 3. Amounts of cleaved TcpA prepilin were quantitated and applied to the formula: % cleavage = (amount of cleaved TcpA in inhibition assay/amount

30   of cleaved TcpA in no inhibitor control assay) x 100. The inhibition of EDAC/Glycinamide is a two step protocol conducted in acidic conditions as described in Example 4. The inhibitors tested and the results are shown below in Table 1.

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TABLE 1 The Effect of Chemical Inhibitors n TcpJ Cleavage *In Vitro*				
Chemical	Specificity	Suggested Concentration	Test Concentration	% Cleavage
E-64	cysteine proteases	(1.4-28 μM)[a] (1-10 μM)[b]	28 μM	96
Calpain inhibitor I	cysteine proteases	45 μM	45 μM	87
NEM	cysteine proteases	1 mM[c]	1 mM	30
Aprotinin	serine proteases	(0.01-0.3 μM)[a]	0.3 μM	100
3,4-DCI	serine proteases	(5-200 μM)[a] (5-100 μM)[b]	100 μM 200 μM	89 54
Pefabloc SC	serine proteases	(0.4-4 mM)[a]	2 mM 4 mM	62 50
Leupeptin	serine and cysteine proteases	1 μM[a] (1-10 μM)[b]	10 μM	85
PMSF	serine and cysteine proteases	(0.1-1 mM)[a] 3 mM[c]	1 mM 3 mM	98 68
Phosphor-amidon	metallo-peptidases	(0.007-0.6 mM)[a]	0.6 mM	89
EDTA-Na	metallo-proteases	(0.5-1.3 mM)[a]	1.3 mM	100
Bestatin	amino peptidase (metallo-)	130 μM[a]	130 μM	81
Pepstatin	aspartic proteases	1 μM[a] (1-5 μM)[b]	1 μM 10 μM 100 μM	95 92 74
EDAC/ Glycin-amide	acid proteases	0.1 M/1 M	0.1 M/0.2 M	0

[a]   Boehringer Mannheim Corp.
[b]   Prolysis, a protease and protease inhibitor Web server,
       Thierry Moreau
[c]   Strom, 1993

The majority of the inhibitors tested had no effect. Included in this group were several inhibitors of cysteine proteases, serine proteases, metalloproteases, and one general commercially available aspartic acid protease inhibitor,

pepstatin. Others had non-specific inhibitory effects such as NEM, which modifies cysteines and PMSF and Pefabloc SC which modifies serines. These inhibitors had partial effects at the higher test doses of the suggested concentrations. The

5 complete insensitivity of TcpJ activity to pepstatin rules out the possibility that TcpJ is a pepsin-like aspartic acid protease. A combination of EDAC and glycine amide, which was selected based upon the active site determination described herein and its known ability to modify aspartic acid residues

10 (Hoare, D.G. and Koshland, D.E. Jr. 1966. *J. American Chemical Society* 88(9):2057-8), inhibited TcpJ peptidase activity in the *in vitro* cleavage assay.

The ability to inhibit type 4 prepilin peptidase activity using peptides corresponding to the region

15 surrounding the cleavage site of the prepilin substrate was also examined. For these experiments, a series of peptides were coexpressed in bacteria in which preTcpA could be processed to the mature form of the TcpJ prepilin peptidase. In order to express only a portion of preTcpA as a peptide,

20 an amber (Am) mutation was created corresponding to position -1, +1, or +5 relative to the cleavage site of TcpA. Peptides were tested that were wild-type, or that contained mutations that would prevent cleavage by TcpJ. Their ability to function *in trans* to compete for TcpJ processing activity of

25 the coexistent wild-type preTcpA was then assessed in a standard western immunoblot which detects precursor and mature forms of TcpA.

The mutations were made in a construct consisting of a 1 kb *tcpJ* fragment followed by a 2 kb *tcpA* fragment on the

30 pALTER-Ex2 backbone. In each of these, *tcpA* contained the following mutations relative to its +1 processed amino acid:

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Plasmid	Amino Acid Substitution and Position							
	-3	-2	-1	+1	+2	+3	+4	+5
pCL1 (wild-type)	Gln	Glu	Gly	Met	Thr	Leu	Leu	Glu
pCL94	Am							
pCL142	Pro							
pCL144			Am					
pCL159			Am					
pCL161	Pro			Am				
pNPD-TCPA-5	Pro		Leu	Am				
pNPD-TCPA-6	Pro			Am				
pNPD-TCPA-7			Leu					Am

To accomplish the analysis, these constructs were electroporated into JM109 cells. Antibiotic resistance on the pALTER-Ex2 backbone was to chloramphenicol, with the exception of CL1 which was to tetracycline. The wild-type TcpA for which processing was assessed was expressed from pGEM-3ZA/TCP-A which confers resistance to ampicillin. Expression of each peptide was driven via the T7 promoter and T7 RNA polymerase expressed upon infection of the cells with λDE3 phage.

Once transformed, the cells in the log-phase of growth were induced by infection with various concentrations of λDE3 phage and the whole-cell protein extract was run out on an acrylamide gel. Western immunoblotting with rabbit αTcpA showed both a leader-sequence processed mature TcpA band (identified relative to a 0395 TCP-A control) and a higher molecular weight unprocessed preTcpA band (identified relative to the pGEM3ZA control). In the experimental lanes, the ratio of unprocessed to processed forms increased with increasing expression of the peptide. These results were observed for every peptide examined, thus demonstrating that multiple peptide derivatives corresponding to the region surrounding the cleavage site of TcpA prepilin are capable of inhibiting

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the activity of the TcpJ type 4 prepilin peptidase. By
"corresponding to" it is meant that the peptide is identical
to the region surrounding the cleavage site of the target
protein or substrate of the TFPP-like aspartyl protease or
5 contains sufficient amino acid similarity such that the
peptide mimics this region. As demonstrated herein, these
peptide mimics inhibit the cleavage activity of the TFPP-like
aspartyl proteases. In a preferred embodiment, these peptide
mimics range in length from approximately 12 to 30 amino
10 acids.

The combined results of the *in vitro* cleavage testing
demonstrated that none of the highly conserved serines or
cysteines are an active site residue of protease activity for
TcpJ. However, D125 and D189 are demonstrated to be protease
15 active site residues of TcpJ, thereby classifying TcpJ as a
monomeric aspartic acid protease. The importance of these two
residue sites is further supported by the data showing that
all substitutions, except of E, at either positions 125 or 189
failed to restore any amount of protease activity indicating
20 both aspartic acids are required for protease activity and
further defining the chemical nature of the reaction.
Therefore, TcpJ is a novel type of protease with two aspartic
acid residues as the active site. This protease is completely
resistant to pepstatin, a general aspartic acid protease
25 inhibitor. The protein sequence analysis of the family of
type 4 prepilin peptidases indicates that a common protease
mechanism exists among these peptidases, regardless of the
bacterial species tested. Further, it is believed that TFPP-
like aspartyl proteases which utilize the same cleavage
30 mechanism and/or contain the same active site as type 4
prepilin peptidase are present in other organisms, including
humans. Such enzymes can be identified by the presence of the
consensus sequence G-X-G-D-(F or V or P or K or I or L or Y)
at the C-terminal aspartic acid residue.

In order to provide further evidence that the aspartic
acid residues corresponding to D125 and D189 of TcpJ comprise
the peptidase active site in all type 4 prepilin peptidases
of the TFPP-like aspartyl protease subclass, mutations were
5   also made to the putative protease active site of aspartic
acid residues D147 and D212 of the other type 4 prepilin
peptidase in *V. cholerae*, VcpD. Mutant D147A and D212A were
constructed in *vcpD* on the plasmid pJM294. Mutant constructs
D147A and D212A were examined for their ability to cleave the
10 TcpA prepilin. Wild-type VcpD is known to be capable of at
least partially cleaving this prepilin. In these experiments,
wild-type, D147A and D212A forms of pJM294 were introduced
into an *E. coli* JM109 strain carrying the TcpA-expressing
plasmid pRTH3G7. Strains were grown to midlog phase; whole
15 cell protein extracts were made from the cultures; and the
extracts were examined by SDS-PAGE and western immunoblot
analysis with anti-TcpA antiserum. Wild-type VcpD cleaved the
TcpA prepilin completely under these conditions. In contrast,
no cleavage occurred in the D147A and D212A mutants. An
20 analogous result was seen with the cleavage of a natural
substrate of VcpD, the type 4 prepilin-like protein EpsI. In
these experiments, wild-type, D147A and D212A forms of pJM294
were introduced into JM290, an *E. coli* JM109 strain carrying
the EpsI(6)His-expressing plasmid, pJM290. Strains were grown
25 to midlog phase under the induction of 1 mM IPTG in order to
express EpsI(6)His. Whole cell protein extracts were made
from the cultures and examined by SDS-PAGE and western
immunoblot analysis using the Tetra-His monoclonal antibody
to detect the c-terminally 6-His epitope tagged EpsI. Wild-
30 type VcpD was able to completely cleave EpsI from the
precursor form to the mature form whereas the mutants were
unable to perform this cleavage. These experiments thus
demonstrate that alanine substitutions at D147 and D212, the
aspartic acid residues in VcpD which correspond to protease
35 active site D125 and D189 of TcpJ, completely eliminate

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peptidase activity. Thus, it is believed that these two residues comprise the active site of all members of the type 4 prepilin peptidase enzyme family.   Accordingly,   by targeting the active site identified herein as comprising two

5   aspartic acid residues, one of skill can identify compounds capable of inhibiting activity of type 4 prepilin peptidases and other TFPP-like aspartyl proteases which utilize this same cleavage mechanism and/or contain the same active site. These compounds are expected to be useful as inhibitors of TFPP-like

10 aspartyl proteases. In one embodiment, these compounds can be used as anti-bacterial agents in the inhibition of the type 4 prepilin proteases. One example of a method for identifying such inhibitors comprises determining the inhibitory activity of a test compound in an *in vitro* cleavage assay. In one

15 embodiment of this assay, a bacterial cell membrane preparation which expresses type 4 prepilin peptidase is incubated with a test compound. The amount of cleavage activity in the preparation in the presence of the test compound is then compared to cleavage activity in a

20 preparation which does not contain the test compound. Test compounds which decrease or inhibit cleavage activity are inhibitors of type 4 prepilin peptidase as well as other TFPP-like aspartyl proteases. For example, using this method, EDAC in combination with glycine amide was identified as an

25 inhibitor of type 4 prepilin peptidase. In addition, peptides corresponding to the region surrounding the cleavage site of the prepilin substrate were also demonstrated to be effective inhibitors of type 4 prepilin peptidase. High throughput screening assays can also be developed in accordance with well

30 known methodologies to identify these inhibitors. Identified inhibitors are believed to be useful as anti-bacterial agents. These agents are also expected to be useful in inhibiting the cleavage activity of other TFPP-like aspartyl proteases in this subclass which utilize the same cleavage mechanism and/or

35 contain the same active site.

Further, knowledge of the chemical nature of test
compounds identified as inhibitors and the target site at
which these inhibitors must act on the enzyme to inhibit
cleavage activity is useful in the identification and/or
5   design and development of additional inhibitory agents. For
example, test compounds with structures known or suspected to
target the aspartic acid residues of type 4 prepilin peptidase
or to mimic peptides corresponding to the region surrounding
the cleavage site of the prepilin substrate can be identified.
10 Alternatively, new test compounds with structures designed to
target aspartic acid residues of type 4 prepilin peptidase or
to mimic peptides corresponding to the region surrounding the
cleavage site of the prepilin substrate can be synthesized.
The ability of these test compounds to inhibit cleavage
15 activity of this peptidase is then determined via well known
methods. In one embodiment, inhibitory activity is determined
via an *in vitro* cleavage assay such as that described herein.
Similar approaches to those used in the design and selection
or identification of protease inhibitors for the treatment of
20 HIV can also be utilized in the design and selection or
identification of these new anti-bacterial agents.

Test compounds identified as inhibitors of peptidase
cleavage activity are believed to be particularly useful as
anti-bacterial agents. Pharmaceutical compositions comprising
25 an active test compound and a pharmaceutically acceptable
vehicle can be formulated in accordance with well known
techniques. The compounds can then be administered to inhibit
virulence factor production by bacteria and to inhibit
bacterial infections in a host. By "host" it is meant to
30 include humans. Further, it is believed that active test
compounds will also be useful in inhibiting development of
drug resistant strains of bacteria when administered in
combination with a second known therapeutically effective
anti-bacterial agent. In this embodiment, it is preferred
35 that the active test compound be administered just prior to

or at the same time as the second anti-bacterial agent. Thus, the present invention also relates to compositions comprising a compound which inhibits type 4 prepilin peptidase activity and a second known therapeutically effective anti-bacterial

5   agent.

        The following non-limiting examples are provided to further illustrate the claimed invention.

EXAMPLES

Example 1: Membrane Preparation

10   Membrane preparation protocol was adapted from the cell fractionation protocol described by Pfau (1998. *J. Bact.* 180(17):4724-33). Overnight cultures were placed on ice for 20 minutes, then pelleted at 5000 x g for 10 minutes. The cell pellet was resuspended in 1 ml 200 mM Tris pH 8.0 to

15 which 1 ml 50 mM Tris pH 8.0, 1 M sucrose, 2 ml water, 20 μl 0.5 M EDTA, and 20 μl lysozyme (10 mg/ml) were added. The cell suspension was allowed to incubate on ice for 30 minutes. Then, 100 μl 1 M $MgSO_4$ was added and the suspension was pelleted at 5000 x g for 10 minutes. The cell pellet was

20 resuspended in 5 ml 50 mM Tris pH 8.0, sonicated 3 times for 15 seconds, and centrifuged at 5000 x g for 10 minutes. The supernatant was then centrifuged at 230,000 x g for 15 minutes. The resulting pellet, which contained the total membrane fraction was resuspended in 200 μl 50 mM Tris pH 8.0.

25 **Example 2: TcpA Prepilin Quantitation**

        Membrane preparations were made from overnight cultures at 42°C of the *E. coli* K38 that does not express TcpA and *E. coli* K38 p3Z-A, pGP1-2 which overexpresses TcpA prepilin. P3Z-A provides *tcpA* under the control of a T7 promoter, and

30 GP1-2 provides the heat-inducible T7 RNA polymerase that drives transcription of the *tcpA* on p3Z-A. Then, 20 μl of the membrane preparation of K38 and K38 p3Z-A, pGP1-2 along with 3 μg of trypsin were subjected to electrophoresis on a 12.5%

SDS-PAGE gel followed by staining with Coomassie Blue. The stained gel was dried using the NOVEX gel drying system. The dried gel was scanned by a Molecular Dynamics Personal Densitometer SI and the densitometry analysis was performed

5 using ImageQuant version 1.2. The volume of the 3 µg trypsin band, the TcpA prepilin band, the corresponding location of the TcpA prepilin band in the TcpA negative control lane (K38), and an approximately 28 kDa band from both K38 and K39 p3Z-A, pGP1-2 were measured while adjusting to a single

10 background value. The mass of the TcpA prepilin present in the K30 p3Z-A, pGP1-2 lane was determined by first subtracting the volume of the TcpA prepilin band from the volume of the corresponding area in the K38 TcpA prepilin-lacking lane. This controlled for the staining intensity of minor protein

15 bands that co-migrate with TcpA prepilin. By comparing the volume values from the 28 kDa bands from the TcpA and TcpA-negative lanes a further adjustment was made to the TcpA prepilin value to control for the loading difference between the lanes. The trypsin band volume was divided by 3 µg to

20 determine a volume/µg value, which was applied to the final TcpA prepilin band value to determine the µg TcpA/µl membrane preparation. The TcpA membrane preparation was diluted with 50 mM Tris buffer to a concentration of 0.2 µg/µl.

Example 3: *In Vitro* Cleavage Assay

25 A membrane preparation from the *E. coli* strain K38 p3Z-A, pGP1-2, which was determined to contain 0.2 µg TcpA prepilin/µl, was the source of prepilin in the *in vitro* TcpJ proteases assay instead of purified prepilin. A membrane preparation of the TcpJ-expressing strain JM109, pCL9, both

30 wild-type and mutant alleles, was the source of the wild-type and mutant TcpJ protein for the *in vitro* cleavage reactions. The 100 µl processing reaction is performed by combining a 50 µl substrate fraction with a 50 µl enzyme fraction and incubating at 37°C for 1 hour. The substrate fraction was

35 prepared by combining 5 µl of the TcpA-containing membrane

preparation (1 μg TcpA), 10 μl 0.5% w/v cardiolipin, 20 μl of
2X assay buffer (125 mM triethanolamine HCl pH 7.5, 2.5% v/v
Triton-X-100), and brought up to the total volume with water.
The enzyme fraction was prepared by combining varying volumes
5   of TcpJ-containing membrane preparation and water to bring the
volume to 50 μl.   The cleavage reaction was stopped by the
addition of 100 μl of 5X protein sample buffer.   An increasing
range of volumes of the membrane preparation containing wild-
type TcpJ was added to the *in vitro* processing assay in order
10 to determine the amount required to cleave 50% of the 1 μg
TcpA included in the assay.   This amount of activity was
defined as 1 unit of TcpJ cleavage activity.

Example 4: Chemical Inhibitors

        Chemical inhibitors were tested for their ability to
15 prevent cleavage of TcpA from the prepilin form to the mature
form in the *in vitro* cleavage assay.   Inhibitors were added
at specific concentrations to the *in vitro* assay mixture,
which contained 1 unit of TcpJ activity.   Inhibitors were
added to both enzyme and substrate fractions so that when
20 combined the inhibitor concentration remained constant.   The
enzyme fraction was allowed to incubate at room temperature
for 30 minutes prior to combination of the two fractions.   The
100 μl reaction was allowed to proceed for 1 hour at 37°C
before addition of 100 μl of 2x SDS protein sample buffer that
25 stopped the reaction.   The specific concentrations of each
inhibitor are described in Table 1.

        The EDAC/glycinamide inhibition protocol is a two step
chemical reaction that modifies carboxylic acid functional
groups, such as those that partly make up the R-group of
30 aspartic acid and glutamic acid.   This protocol is based on
well known procedures set forth for the selective modification
of carboxyl groups in proteins.   TcpJ containing membranes
containing the equivalent of 6 units of TcpJ activity were
incubated in 80 μl with 25 mM potassium biphthalate, pH 4.0,
35 and 100 mM EDAC (1-ethyl-3-(3-dimethlaminopropyl)carbodiimide

hydrochloride) for 30 minutes at room temperature. Twenty
microliters of 1 M glycinamide was added to the mixture and
another 30 minute incubation at room temperature was performed
before the modification reaction was stopped by the addition
5 of 150 μl of 200 mM Tris buffer, pH 8.   The mixture was
dialyzed against 50 mM Tris buffer, pH 8, for 1 hour and then
centrifuged at 230,000 x g (60,000 rpm in TLA 100.3 rotor) for
15 minutes. The resulting membrane pellet, which contains the
chemically modified TcpJ, was resuspended in 60 μl 50 mM Tris,
10 pH 8.   Twenty microliters of the membrane suspension was
subjected to the *in vitro* peptidase assay to determine the
peptidase activity.   Activity of the modified TcpJ was
compared to an equivalent amount of TcpJ that had been
subjected to a mock modification procedure which did not
15 include EDAC or glycinamide.